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The Pathophysiology of Nitrogen Dioxide During Inhaled Nitric Oxide Therapy

Priscilla C. Petit,* David H. Fine,* Gregory B. Vásquez,* Lucas Gamero,* Mark S. Slaughter,† and Kurt A. Dasse*

Administration of inhaled nitric oxide (NO) with the existing compressed gas delivery systems is associated with unavoidable codelivery of nitrogen dioxide (NO_2), an unwanted toxic contaminant that forms when mixed with oxygen. The NO_2 is generated when NO is diluted with O_2-enriched air before delivery to the patient. When NO_2 is inhaled by the patient, it oxidizes protective antioxidants within the epithelial lining fluid (ELF) and triggers extracellular damage in the airways. The reaction of NO_2 within the ELF triggers oxidative stress (OS), possibly leading to edema, bronchoconstriction, and a reduced forced expiratory volume in 1 second. Nitrogen dioxide has been shown to have deleterious effects on the airways of high-risk patients including neonates, patients with respiratory and heart failure, and the elderly. Minimizing codelivery of NO_2 for the next generation delivery systems will be a necessity to fully optimize the pulmonary perfusion of NO because of vasodilation, whereas minimizing the negative ventilatory and histopathological effects of NO_2 exposure during inhaled NO therapy. *ASAIO Journal* 2017; 63:7–13.

Key Words: nitrogen dioxide (NO2) exposure, inhaled nitric oxide, pulmonary hypertension, oxidative stress, right heart failure

Patients with pulmonary hypertension (PH) or heart failure tend to have high levels of oxidative stress (OS), which contributes to the eventual fibrosis seen in the lungs and hearts of these patients.[1] OS has been suggested to serve as the root cause of heart failure and is exacerbated in patients undergoing surgery for cardiac transplantation or placement of a left ventricular assist device (LVAD).[2,3] Traditional use of inhaled nitric oxide (NO) lowers pulmonary pressures to lessen the risk of right heart failure (RHF). Lowering pulmonary pressure also helps to reduce the mitochondrial metabolic rate in the right ventricular myocytes and, therefore, OS.[2]

In addition to inducing pulmonary vasodilation, inhaled NO may be used to modulate the biochemical basis of OS, which has been implicated to play a key role in the onset of RHF. Focusing on reducing supplemental oxygen, reducing the use of inotropes, lowering pulmonary pressures, and minimizing OS may prove beneficial in mitigating the need to use right ventricular assist devices in LVAD and cardiac transplant patients.

NO diluted in nitrogen (N_2) is currently supplied from a tank of compressed gas. The major source of nitrogen dioxide (NO_2) that is codelivered to the patient comes from the formation in the delivery equipment and from the rapid reaction of NO with O_2, especially during the dilution step. The amount of NO_2 delivered to the patient must be kept to trace amounts under all circumstances to avoid serious adverse events.

The purpose of this article is to review the pathophysiology associated with codelivery of NO_2 and the relationship of elevated NO_2 to OS. A better understanding of this relationship has the potential to lead to improved methods for medically treating patients with PH or heart failure and the possibility of elucidating the biochemical basis contributing to the onset of RHF during and after the implantation of a LVAD.

Mechanisms of Action for Nitric Oxide

NO, a naturally occurring signaling molecule generated in endothelial cells, diffuses into the vascular smooth muscle to cause vasodilation.[4,5] When inhaled into the lungs to treat PH, NO reduces mean pulmonary arterial pressure as well as pulmonary vascular resistance in patients that are vasoreactive.[5]

Inhaled NO reduces and prevents PH for patients undergoing cardiac surgery, including those with PH at risk of right ventricular dysfunction during and after the implementation of left ventricular mechanical circulatory support.[1] Inhaled NO has also been shown to be an effective pulmonary vasodilator in patients with congenital heart disease, valvular heart disease, post heart transplantation, and adult respiratory distress syndrome.[8,10]

The vasodilatory effect of inhaled NO is limited to well-ventilated areas of the lung, where NO selectively dilates vessels.[5] Limiting the vasodilatory effects of NO to better ventilated regions of the lung results in an improvement in ventilation–perfusion (V/Q) matching. This is in contrast to systemic vasodilators that dilate most of the pulmonary vessels, including the poorly ventilated areas, resulting in potential V/Q mismatch. An additional advantage of the localized effects of NO is the lack of systemic hypotension.

From the *Biomedical Research, GeNO LLC, Cocoa, Florida; and †Department of Cardiovascular and Thoracic Surgery, University of Louisville, Louisville, Kentucky.

Submitted for consideration March 2016; accepted for publication in revised form July 2016.

Nitric Oxide is only approved for use by the Food and Drug Agency (FDA) for neonates with hypoxic respiratory failure because of persistent pulmonary hypertension of the newborn. Additional indications described in this manuscript are not approved for use by the FDA and are considered off label. Inhaled nitric oxide is approved for inhalation as a vasodilator, which, in conjunction with ventilation and other appropriate agents, treats term and near-term newborns (>34 weeks gestation) with hypoxic respiratory failure associated with evidence of pulmonary hypertension.

Disclosures: Priscilla C. Petit is an employee of GeNO LLC.

Correspondence: Priscilla C. Petit, Biomedical Research, GeNO LLC, 2941 Oxbow Circle, Cocoa, FL 32926. Email: priscilla.petit@genollc.com.

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DOI: 10.1097/MAT.0000000000000425

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The use of low-concentration inhaled NO can prevent, reverse, or limit the progression of disorders, which can include acute pulmonary vasoconstriction, traumatic injury,[13] aspiration or inhalation injury,[6] fat embolism in the lung,[7] acidosis,[8] inflammation of the lung,[9] adult respiratory distress syndrome,[10] acute pulmonary edema,[11] acute mountain sickness,[12] post cardiac surgery,[14,26] acute PH,[15] persistent PH of the newborn,[16] perinatal aspiration syndrome,[17] acute pulmonary thromboembolism,[7,24] heparin–protamine reactions,[18] sepsis,[19] asthma,[20] and sickle cell anemia.[21] NO can also be used to treat chronic PH,[22] bronchopulmonary dysplasia,[23] chronic pulmonary thromboembolism,[24] and chronic hypoxia.[25]

Historical Preclinical and Clinical Observations Related to Toxic NO_2

Inhaled NO received FDA approval under a New Drug Application 505(b)(2) pathway in 2000. Nitric oxide was approved to treat neonates (>34 weeks) with hypoxic respiratory failure associated with clinical or echocardiographic evidence of PH to mitigate the need to treat these patients with extracorporeal membrane oxygenation.[17]

NO acts intracellularly through the regulation of the NO synthase pathway. In contrast, any NO_2 that is codelivered with inhaled NO exerts its effects extracellularly. This occurs because NO readily crosses the cellular membrane, whereas NO_2 has a limited ability to cross the membrane.

The lungs are at risk for the highest degree of oxidative damage because of direct inhalation of oxygen. To mitigate this risk, the lungs have abundant protective mechanisms against oxidative damage by containing a high concentration of antioxidants. Inhalation of NO_2 also places the lungs at risk by producing cellular injury and oxidation of both epithelial fluid lining (ELF) and epithelial components before the onset of an inflammatory response.[27]

NO_2 is a toxic gas that forms nitric and nitrous acids upon contact with moisture. It can damage the lungs in three ways: 1) it is converted to nitric and nitrous acids in the distal airways, which directly damages certain structural and functional lung cells; 2) it initiates free radical generation, which results in protein oxidation, lipid peroxidation, and cell membrane damage; and 3) it reduces resistance to infection by altering macrophage and immune function.[28]

Low concentrations of NO_2 gas may initially cause mild shortness of breath; then, after a period of hours to days, victims may suffer bronchospasm and pulmonary edema. Exposure to higher (>10 ppm) concentrations of NO_2 gas may induce an immediate response in a subject that may include coughing, fatigue, nausea, choking, headache, abdominal pain, and difficulty breathing. A symptom-free period of 3 to 30 hours may then be followed by the onset of pulmonary edema with anxiety, mental confusion, lethargy, and loss of consciousness. If the patient survives, the episode may be followed several weeks later by bronchiolitis obliterans. Inhalation of very high concentrations (>50 ppm) can rapidly cause burns, spasms, swelling of tissues in the throat, upper airway obstruction, and death.[28]

Absorption of NO_2 can lead to tachycardia, a dilated heart, chest congestion, and circulatory collapse. Obstruction of the bronchioles may develop days to weeks after severe exposure. Patients may also suffer potential hemorrhage of the lungs and respiratory failure.[28]

Children are more vulnerable to NO_2 than adults because of the relatively smaller diameter of their airways. Children may also be more vulnerable because of relatively increased minute ventilation per kilogram of body weight.[28]

Van Meurs *et al.*[29] published results in the *New England Journal of Medicine* in 2005 on the use of inhaled NO in premature infants with severe respiratory failure. They reported that at NO dose levels of 5 to 10 ppm, NO_2 concentrations were at least 3 ppm in four infants and 5 ppm in two infants. In contrast, no infants in the placebo group had elevated NO_2 concentrations in this study.[29,30]

There is substantial clinical evidence in healthy volunteers and in patients with significant lung disease for potential adverse effects at low levels of NO_2.[29,31] The evidence provided by Van Meurs *et al.*[29] suggests that NO_2 is harmful in neonates with significant comorbidities, and that reducing the NO_2 concentration would provide greater safety to that patient population.

NO_2 Effects in Animals and Humans

Research in animals and humans demonstrates the negative effects of both acute and chronic NO_2 exposure at concentrations above 1 ppm. These effects are present at the cellular level before symptoms manifest clinically.

The majority of data for the harmful effects of NO_2 are found in environmental publications. The WHO produced a summary of air pollutants in 1987 that was revised in 2005 that includes comprehensive summaries of nonclinical and clinical data developed on NO_2.[31]

Additionally, the Dutch Expert Committee on Occupational Standards in 2004 conducted a thorough review of relevant nonclinical and clinical studies with NO_2 exposure and provided recommended exposure limits for humans.[30]

The US EPA has set 0.053 ppm, averaged annually, and 0.100 ppm, averaged more than 1 hour, as the two primary NO_2 levels that are regulated in the US.[32] Populations that are currently at risk of exceeding the EPA primary standards are adult patients with respiratory or heart failure being treated with inhaled NO as well as neonates treated for persistent PH of the newborn (PPHN).

NO_2 Effects in Animals

Both acute and chronic NO_2 exposures have a demonstrated effect in pulmonary metabolism, pulmonary structure, pulmonary function, airway inflammation, and susceptibility to bacterial and viral infections.[30,31]

Morphological changes in the lungs were revealed after NO_2 exposure in animals consistent with more severe defects resembling hyperplasia, fibrosis, or emphysema, of which the last two are known to be nonreversible. These and other effects, such as lowered pulmonary host defense, were consistently found at 0.5 ppm and higher in animals.[30]

Other studies have demonstrated changes in lipid peroxidation, NO_2 pulmonary antioxidant enzyme activities, and lung morphology with NO_2 exposure ranging from 1 to 20 mg/m^3. Furthermore, nonclinical studies have shown that NO_2 levels of 2 ppm or higher can produce alveolar cell hyperplasia,

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altered surfactant hysteresis, changes in the epithelium of the terminal bronchiole, and loss of cilia in the airways of rats.[29,33]

NO_2 Effects in Humans

Health effects after a single exposure of NO_2 for patients with chronic obstructive pulmonary disease (COPD) and asthma have shown that NO_2 is harmful to the upper and lower respiratory tract including the bronchioli and alveoli.[30] These effects include lowered pulmonary host defense shortly after the exposure was stopped.

The no-effect threshold was determined to be at 0.5 ppm NO_2.[30] As explained in the WHO publication, the lowest level of NO_2 exposure reported in more than one laboratory to show a direct effect on pulmonary function in asthmatics, when exposed for 2–2½ hours, was 0.3 ppm.[34–36] These observations must be considered within the context of neonates with PPHN and significant comorbidities. The concentration of NO_2 with a harmful effect in this vulnerable patient population may well be lower than what was observed clinically in healthy volunteers.

According to a WHO publication on the effects of NO_2 on airway inflammation at a cellular level, NO_2 was found to enhance epithelial damage and increase baseline smooth muscle tone.[31] Moreover, the publication characterizes NO_2 concentrations from 3 to 5 ppm as high and describes the deleterious effects that can result from such concentrations on cellular and inflammatory mediators.

Extracellular Damage Associated with NO_2

Extracellular NO_2-induced damage starts when gaseous NO_2 enters the lungs and interacts with the ELF. The ELF contains aqueous extracellular antioxidants including glutathione (GSH) and ascorbic acid. Interaction between NO_2 and these antioxidants leads to the production of reactive oxygen species (ROS) including superoxide and hydrogen peroxide. The extracellular ROS serve as the mediators of cellular injury seen in the airways and surrounding the alveoli of the lungs as a result from inhaling NO_2 (**Figure 1**).

Biochemical Mechanism of NO_2 Injury Through Oxidative Damage

NO_2 has been implicated in the etiology of oxidative damage.[39] Although oxidation reactions are essential for life, they can also be damaging; animals maintain complex systems of multiple types of antioxidants, such as vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, may contribute to OS.[40]

OS reflects an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage proteins, lipids, and DNA. Oxidative stress from oxidative metabolism causes base damage as well as strand breaks in DNA. Further, some ROS are essential for normal mechanisms of cellular signaling.[41]

OS is suspected to be important in neurodegenerative diseases including Lou Gehrig's disease and multiple sclerosis.

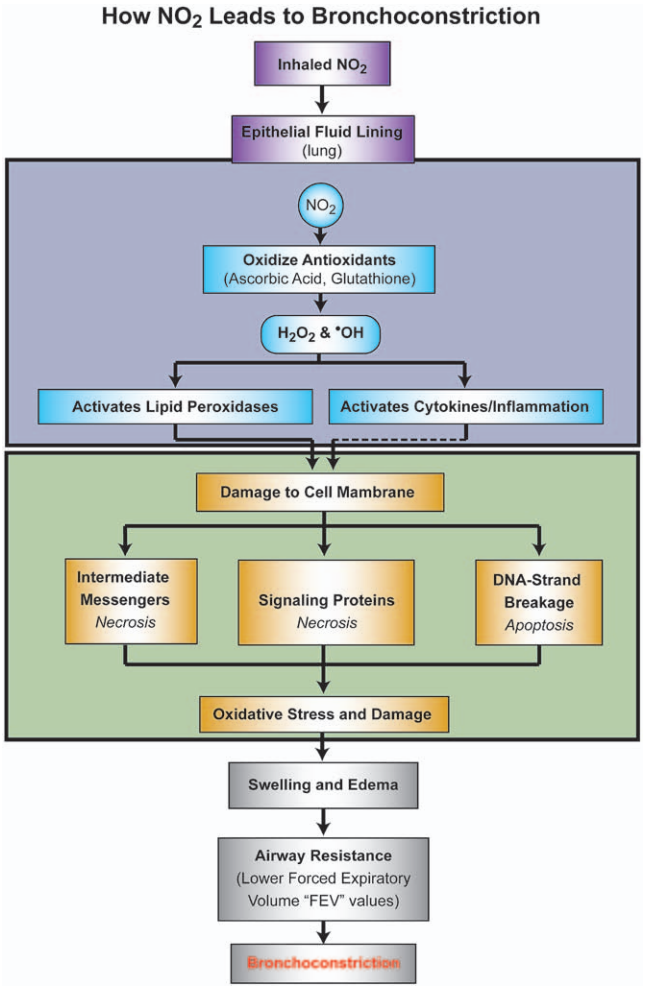

Figure 1. How NO_2 leads to bronchoconstriction. NO_2, nitrogen dioxide. full color online

Evidence via biomarkers of ROS and reactive nitrogen species (RNS) indicates that oxidative damage may be involved in disrupting mitochondrial respiration and mitochondrial damage related with Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases.[42]

OS is linked to cardiovascular disease because oxidation of low-density lipoprotein (LDL) in the vascular endothelium is a precursor to plaque formation.[43] OS also plays a role in the ischemic cascade because of oxygen reperfusion injury after hypoxia.[44] This cascade includes both strokes and heart attacks.[45] OS has also been implicated in chronic fatigue syndrome,[46] hyperoxia,[47] and diabetes.[48] OS can be reduced by avoiding exposure to unnecessary oxidation,[49] reducing mitochondrial metabolism,[50] and by increasing the use of antioxidants.[51,56]

The biochemical environment in the lung alveoli is complex, because of the variation of protective proteins, antioxidant concentrations, pH, and partial oxygen pressure across the lungs. Each of these factors influences the protective pathways and resultant oxidative byproducts. The lungs are at the greatest risk for oxidative damage because of the presence of exogenous oxygen. To mitigate this risk, the lungs also have the most abundant protective mechanisms against oxidative damage including an ELF that contains GSH, superoxide

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dismutase, catalytic iron, catalase, uric acid, and surfactant unsaturated fatty acids (UFAs). Although these molecules are often found intracellularly, those found in the ELF are extracellular isozymes that differ from their corresponding intracellular counterparts to function in the extracellular environment.

The studies of Velsor *et al.*[27] found a negligible contribution of superoxide dismutase, catalase, uric acid, and UFAs related to the clearance of NO_2 in the ELF. The primary reactions with NO_2 were determined to be limited to GSH and ascorbic acid. These interactions were determined to be dependent upon pH, partial oxygen pressure, and the concentration of the reactive components.

Impact of Nitric Oxide Dose on NO_2 Levels

The homogeneous gas-phase reaction of NO with O_2 that produces NO_2 is first order[37] in O_2, second order in NO, and third order in total pressure.

$$NO + NO + O_2 \xrightarrow{K} NO_2 + NO_2$$

Typically, NO is shipped in cylinders of compressed gas, diluted in N_2 to a concentration of 800 ppm. The NO is mixed and diluted with O_2-enriched air[52] just before use, to provide NO at the appropriate therapeutic dose. As the mixing proceeds, the local concentration of NO is diminished until, at equilibrium, it reaches the target therapeutic concentration. Because the rate of formation of NO_2 is dependent upon the square power of the NO concentration, and linearly with the oxygen concentration and time, the amount of NO_2 that can be formed during the mixing process can be considerable.[53–55]

Schedin *et al.*[54] observed that the rate of formation of NO_2 at 80 ppm NO, and 85% oxygen was approximately 10-fold higher than would be calculated when they inserted the target therapeutic NO concentration in the kinetic equation. They concluded that mixing point generation of NO_2 was a major source of NO_2 in hyperoxic gas mixtures even in rapid delivery systems. Lindberg and Rydgren[55] reported an "undescribed and unexpected finding" in flow experiments when 1,000 ppm of NO in N_2 was diluted down to 50 or 100 ppm of NO with air or oxygen. They found that 90% of the NO_2 that was measured at 0.5 seconds was actually present at time zero. Nishimura *et al.*[53] reported that when using a ventilator to mix NO and air, there was very little NO_2 formed if the NO was first diluted with N_2 instead of air before it was mixed with oxygen in the ventilator.

There are three distinct sources for the NO_2 that reaches the patient: 1) NO_2 that is present as a preformed contaminant in the gas cylinder, which is negligible. 2) NO_2 that is formed *in situ* as the NO is diluted and mixed with O_2-enriched air to achieve the intended dose. This source is often underestimated because the NO concentration that is used in the kinetic equation is assumed to be the target therapeutic concentration, instead of the instantaneous NO concentration as the mixing proceeds. For a 800 ppm NO source, at the moment of mixing, the rate of NO_2 formation is 1,600 times faster than at the target therapeutic dose of 20 ppm NO. This source is typically at least 10 times greater than the other two sources combined. 3) NO_2 formation in the gas lines to the patient after the therapeutic NO concentration has been achieved.

The lower the starting NO concentration, the lower the NO_2 concentration that is delivered to the patient. Thus, if the target therapeutic NO concentration was 20 ppm, then the NO_2 level could be reduced 16-fold using a gas source of 200 ppm *versus* 800 ppm NO.

Triggers and Consequences of Oxidative Stress

NO_2 damage occurs when the reaction leads to breakdown products of the aqueous antioxidants. These breakdown products trigger cytokine activation, inflammation, as well as intracellular OS in the cells lining the airway. This results in an increase in airway resistance as determined by a decrease in the forced expiratory volume over 1 second (FEV_1).[65] A decrease in FEV_1 represents a diminished therapeutic benefit of inhaled NO. Selected drugs may be prescribed for patients at risk for OS to manage inflammation and fibrosis.

Among other triggers that lead to OS, one appears highly relevant to heart failure and PH. Agents that induce a positive inotropic effect can lead to an increase in oxygen consumption and induce OS because of an increase in mitochondrial metabolic activity.[2] An increase in mitochondrial metabolic activity may lead to excess production of superoxides that participate in the formation of peroxynitrite, which, over time, leads to fibrosis.

Conditions that lead to an increase in transforming growth factor-B (TGF-B) have been shown to lead to profibrotic activity. Peroxynitrite activates the TGF-B pathway, and the activation of TGF-B may also stimulate connective tissue growth factor which leads to fibrosis.[57] Given the history of chronic heart failure and PH patients, OS may set the stage for the onset of RHF and PH and further contribute to complications associated with reperfusion injury.

OS has been directly implicated in RHF with an effect on right ventricular myocytes.[2] OS can be exacerbated by reperfusion injury after cessation of cardiopulmonary bypass when transitioning from a low to high cardiac output state.[44] Given this background, the management of RHF may not only include the use of pulmonary vasodilators to reduce the right ventricular afterload but may also require a strategy that attempts to directly minimize the extent of OS associated with this surgical procedure.

Physiological Mechanisms Related to Reactive Oxygen Species Injury to the Lung Epithelia

ROS are formed as a natural product of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times, when supplemental oxygen is used or during increased metabolic stress, ROS levels can increase dramatically and cause significant damage to cell structures.

Barth *et al.*[58] showed that NO_2-induced pulmonary vascular damage in animals was both concentration and time dependent. NO_2 was found to injure the pulmonary epithelium causing cellular damage and inflammation.[58–60] This process activates cytokine release, and the resultant inflammatory cascade commences. The inflammatory response leads to OS producing superoxide, peroxides, hydroxyl radicals, and peroxynitrite, if NO is present.[38]

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NO_2 has been shown to cause alveolar epithelial apoptosis *in vitro*. Nitrogen dioxide increases alveolar septal cell turnover leading to accelerated lung growth *in vivo*.[60] This study found that the lung growth was associated with an imbalance of the extracellular matrix composition, specifically a decreased collagen to elastin ratio. The lung epithelial barrier integrity in rats was shown to be altered by NO_2 exposure, increasing protein permeability in lung endothelial and epithelial cells. In addition to epithelial damage to the airways, NO_2 was found to increase the tone of respiratory smooth muscle.[59–61]

In summary, NO_2 in the lung triggers the formation of ROS, which causes cellular injury that, in turn, triggers a cytokine response that results in OS and inflammation. The OS turns on both the formation of ROS and RNS leading to peroxynitrite formation. The formation of peroxynitrite can then induce apoptosis or necrosis, which both damage the airways and lungs[38] (**Figure 2**).

Cost Considerations

Inhaled NO is an effective but costly therapy.[62] In an era of increased awareness regarding health care spending, the cost of NO must be reduced to encourage prophylactic use. Acknowledgment of its high cost is evidenced by several studies conducted to decrease direct costs associated with its use.[63] Expanding the use of inhaled NO is likely to drive the cost of NO down, making it more attractive to intervene earlier and hopefully improve outcomes. Next generation delivery systems along with a purer drug are anticipated to be less expensive, to be responsive to users, and the cost conscience health care market.

Future Directions of Inhaled Nitric Oxide Delivery

To summarize, NO_2 has been shown to have deleterious effects on the airways of high-risk patients including neonates, patients with respiratory and heart failure, and the elderly. Asthmatic patients are particularly vulnerable. In addition, NO_2, because of its role as a reactive N_2 species and the relationship to ROS, may trigger the OS cascade with all of the inherent risks associated with this pathway. A key consequence of OS is the activation of profibrotic pathways leading to fibrosis in both the heart and lungs. Nitrogen dioxide has also been shown to directly activate pathways leading to fibrosis.

Patients undergoing heart transplantation and LVAD implantation are certainly at high risk of these adverse effects. The current users of inhaled NO may ignore, be complacent, or are unaware of the negative clinical sequelae associated with exogenous NO_2 levels. Eliminating the codelivery of NO_2 could lead to *lower doses* required for the beneficial effects of inhaled NO. This occurs by negating the ventilation/perfusion effects of NO_2 and possibly lead to the ability to finally demonstrate a survival benefit of this therapeutic drug if applied prophylactically, before cardiac surgery, rather than used as a rescue therapy.

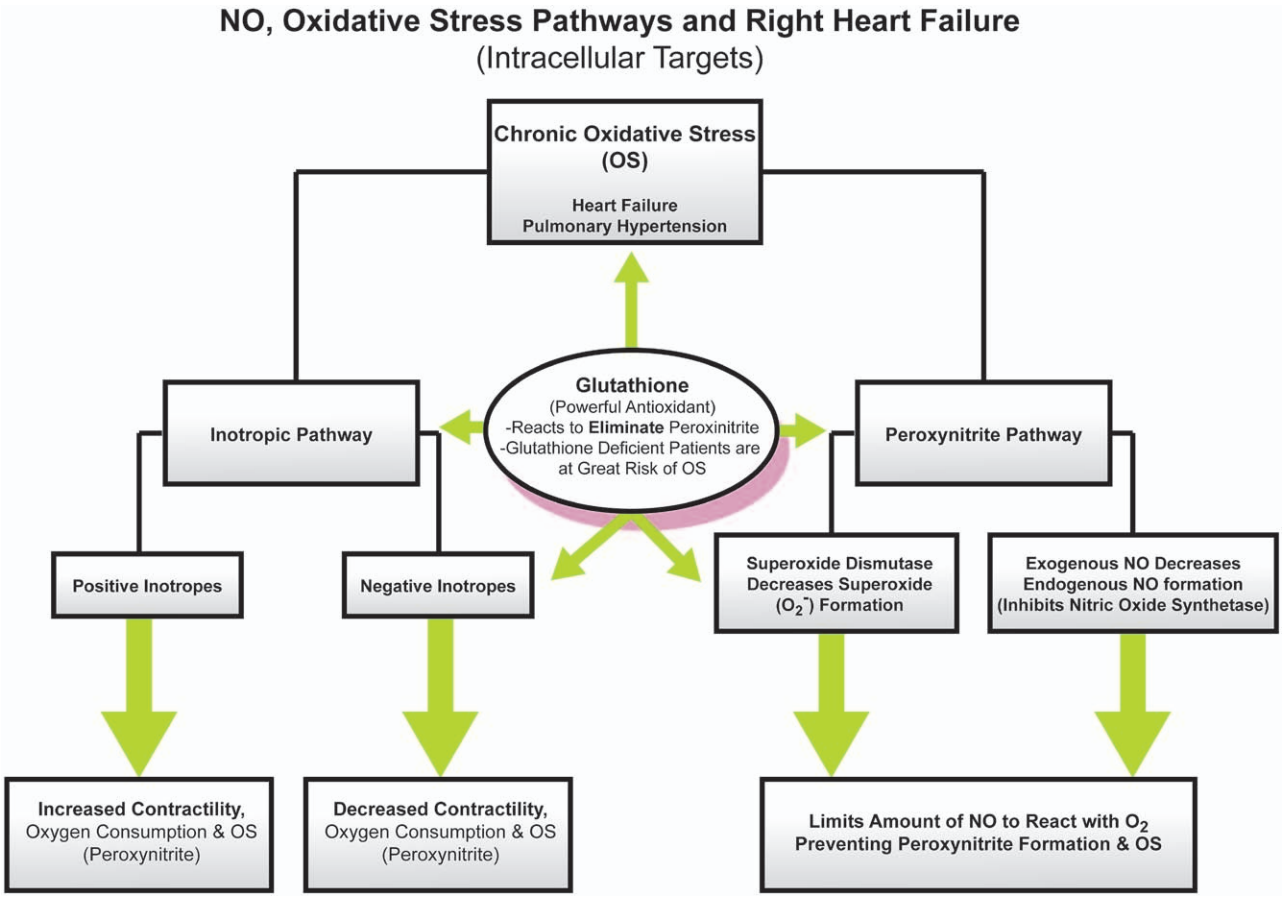

Figure 2. NO, OS pathways, and RHF. NO, nitric oxide; OS, oxidative stress; RHF, right heart failure. full color online

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Avoidance of scenarios leading to OS and RHF may require the use of inhaled NO preoperatively for several days in advance of the operative procedure. Preoperative use of NO may lower the degree of OS rendering the patient a better candidate for surgery. The use of NO should also be considered for use intraoperatively, and for a short period postoperatively, to minimize complications associated with PH, reperfusion injury, or the unanticipated sudden onset of RHF.[64]

The inhaled NO concentration must be kept at the minimally effective dose to reduce the formation of NO_2. Next generation delivery systems should focus on limiting both the level of NO_2 delivered to the patient and keeping methemoglobin levels as low as possible.

The use of inhaled NO may be used to achieve the following goals in medically managing the patient:

1. Inhaled NO can reduce supplemental oxygen requirements. Supplemental oxygen increases the risk of OS by increasing superoxides produced in the mitochondria.
2. Superoxides (free radical) can be further reduced by minimizing the use of inotropes to lower myocyte mitochondrial metabolic activity.
3. Metabolic demand can be managed by reducing pulmonary pressures through the vasodilatory effects of inhaled NO. Reducing pulmonary pressure will be effective by lowering right heart pressure and may set the stage for a smoother postoperative course.

By addressing these concerns, it may be possible to treat the hemodynamic concerns, as well as the underlying biochemical and pathophysiological factors that contribute to the sudden intraoperative onset of right heart dysfunction postoperatively. Further exploration of the underlying biochemical changes contributing to RHF may also lead to better predictors of patients at risk of requiring intervention with a right ventricular assist device.

Future randomized controlled studies are encouraged to reassess the optimal therapeutic dose of inhaled NO when delivered in a purer form with minimal codelivery of NO_2. Future studies should also explore the use of NO with minimal NO_2 contamination to preemptively inhibit the systemic consequences of OS. Such studies could also examine the use of NO alone or combined with other available vasodilators and pharmaceuticals used to minimize or mitigate the OS.

Acknowledgment

A special thanks to Isabelle Kemp, MS, for her research contributions to this paper.

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